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A study of possible toxicological effects of Methylparaben on estrogen receptor-α negative MCF-7 human breast cancer cell line

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Abstract
Aim: Methylparaben (MP) is the most commonly used preservative in various everyday life products. It is considered one of the xenoestrogens because of its metabolic activity as an estrogen agonist. Hence, it is implicated to play a role in breast cancer development. The human breast adenocarcinoma cell line MCF-7 (Michigan Cancer Foundation-7) is used as a standard model for in vitro cancer research. However, it is now recognized that MCF-7 is heterogeneous concerning both the expression of hormone receptors and the utilization of the signaling pathways linked to these receptors. The present study aimed to study in vitro toxicological effects of MP on estrogen receptor- negative (ER–ve) MCF-7 breast cancer cell line.
Material and Methods: MCF-7 cells were verified immunologically for the presence/absence of ER using immunocytochemistry and flow cytometry. MCF-7 cells were incubated with five MP at serial concentrations of 4×10^{-5} M, 6×10^{-5} M, 8×10^{-5} M, 1×10^{-4} M and 2×10^{-4} M. The effect of a single exposure to serial concentrations of MP was studied concerning the proliferation of ER-ve MCF-7 cells. Cell viability was determined using MTT assay.
Results: A single exposure to the experimented doses of MP did not demonstrate any stimulatory effect on proliferation of MCF-7 cells with no significant differences between the doses compared to untreated cells. Simultaneously, the applied doses exerted no effect on alpha ER expression.
Discussion: In conclusion, a single exposure to MP in various dose ranges did not influence estrogen -ve MCF7 cancer breast cells' growth.

Keywords
Methylparaben; Breast cancer; ER-ve MCF-7; Flow cytometry

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Introduction

Parabens (4-hydroxybenzoic acid esters) are common ingredients in thousands of processed food, pharmaceuticals, cosmetics, and daily personal care products [1,2]. Since the human consumption of these compounds is frequent through the exposure to the skin, lips, eyes, oral mucosa, nails, and hair, researchers have investigated whether the three most commonly-used parabens (methyl-, ethyl- and propylparaben) can be considered as xenoestrogens; a class of endocrine-disrupting substances [3].

ERα is a major ER sub-type that plays a crucial role in breast cancer progression. ERα mediates estrogen-induced cell proliferation in ERα-positive breast cancer cell lines in an autocrine mode [4]. The molecular mechanisms by which ERα mediates estrogen-induced cell proliferation in ERα-positive breast cancer cell lines are not clear. It was found that ERα promotes S–phase entry of MCF–7 cells and cellular proliferation, while E2 enhances ERα-induced proliferation of MCF–7 cells by stimulating the expression of proliferating cell nuclear antigen (PCNA) and Ki–67 [5].

Molecular modeling showed that paraben molecules bind into the ligand-binding pocket of the crystal structure of the ligand-binding domain (LBD) of ERα in place of 17β-estradiol [1]. In vivo studies implicated that estrogenic chemicals are capable of binding to ER, activate estrogen-responsive gene expression and subsequently enable the proliferation of estrogen-dependent cells that increase uterine weight in the immature or ovariectomized rodent assay [6].

Notwithstanding, the majority of the studies addressing interferences of parabens with estrogen hormone action focused on the ER-positive status. The possible interferences of parabens with pre-receptor control enzymes modulating endocrine functions remain to be investigated [2]. Moreover, MCF-7 cell lines are recognized as heterogeneous with respect to both the expression of hormone receptors and the utilization of the signaling pathways linked to these receptors, resulting in their phenotypic heterogeneity [7].

In an attempt to elucidate the controversies about parabens as breast cancer promoters, the present study aimed to study in vitro toxicological effects of methylparaben on ERα negative MCF-7 human breast cancer cell lines.

Material and Methods

The study was conducted at the Center of Excellence for Research in Regenerative Medicine Applications (CERRMA), Alexandria Faculty of Medicine. The experimental protocols were approved by the Medical Ethics Committee (IRB NO: 00012098-FWA- NO: 00018699), Faculty of Medicine, Alexandria University, Egypt.

Chemicals:

Stock solution of methylparaben in 0.1 Methanol (MP/ H5501, minimum purity 99.0% purity, Sigma Aldrich, Egypt/ molecular weight 152.149 g/mol. For the cell culture experiment, five serial concentrations were prepared 4×10^{-5} M, 6×10^{-5} M, 1×10^{-4} M, 2×10^{4} M, taking into consideration that the concentration of ethanol was ≤ 0.1% to ensure cell viability [8].

Cell culture:

MCF-7 (human breast cancer cell line, HTB-22) was obtained from VACSERA - Cell Culture Unit, Dokky, Giza, Egypt. Cells were grown as monolayer cultures in RPMI-1640 complete media (Roswell Park Memorial Institute, Lonza) containing 300mg/L L-glutamine,100 IU/ml penicillin, 100 µg/ml streptomycin (Lonza) and 10% fetal bovine serum (FBS, Lonza). The cells were incubated at 37 °C 5% CO2 atmosphere (Thermofisher CO2 incubator). Cells were continuously monitored (Olympus BX 40 phase contrast inverted microscope) for morphology and confluence. On reaching 80-90% confluence, the cells were subcultured with a suspension with 0.25% (w/v) trypsin EDTA (Lonza), then plated in different culture vessels [9].

Immunocytochemical analysis:

The trypsinized cells were centrifuged, the supernatant cells were removed and the cell pellet was re-suspended in 1ml of phosphate buffer saline (PBS). Semiautomated rotatory microtomed sections with a thickness of 2 µm thick were mounted on positively charged slides. The expression of ER was assessed using mouse monoclonal antibodies (ER- alpha Monoclonal Antibody SP1 1:10/ Invitrogen) with the application of the universal peroxidase-labeled streptavidin-biotin technique. 3,3 diaminobenzidine was applied as a chromogen for antibody detection and was counterstained with Mayer's hematoxylin. The negative control was a section of the same sample where the primary antibody was replaced by PBS. Breast adenocarcinoma, which previously has shown negative immunostaining of estrogen receptors, was considered positive controls [10].

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay for Cell viability:

Cells were seeded in 96-well flat-bottom plates at an initial concentration of 5000 cells per well and incubated for 24 hours, after which the media was replaced with phenol red-free media with 5% charcoal-stripped FBS (Fetal bovine serum) (Biowest) containing the different five concentrations of methylparaben and incubated for seven days. On the 7th day, the media was replaced by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution and incubated for four hours.

The media was removed and 100 µl DMSO/well was added and gently rocked in the dark for 20 minutes to dissolve formazan crystals. ELISA plate reader (Tecan, Infinite F50) was applied to detect the absorbance at a wavelength of 570 nm. The percentages of cell viability were calculated as the ratio of treated to control untreated cell absorbances after blank correction. All the samples were run in triplicate [11].

Cell proliferation assay:

Cells were seeded in a monolayer at a density of 10,000 cells/ well into 24 well plastic culture plates, incubated for 24 hours, followed by media replacement with phenol red-free media with 5% charcoal-stripped FBS. The five different concentrations of MP were added and incubated for 7 days. On the seventh day, the trypan blue exclusion test was applied using a Neubauer hemocytometer slide to assess the number and viability of MCF7 cells (100 µl with a 0.4% trypan blue solution / Sigma-Aldrich, St. Louis, USA). Manual cell count was done by examining the stained (non – viable cells) under the inverted microscope. The calculation of the number of non-viable cells was obtained as average cell count/square and multiplied by dilutional factor x 10^{4}). All the samples were run in triplicate [9].

Quantitative assay for ER expression by Flow cytometry:

To evaluate the expression of the ER receptors of the treated MCF-7 cells relative to control, triplicates of cell samples were plated in 6-well plates at a density of 2 x 104cells/ well. After incubation for 24 hours, the media was replaced with phenol red-free media with 5% charcoal-stripped FBS, including 40 up to 200 µM concentrations of MP, and incubated for seven days. After seven days, the culture medium was removed from the wells, the cells were washed twice with sterile PBS, and a 0.25% trypsin-EDTA solution was added to detach the cells. After 5 min incubation at 37 °C and 5% CO2, fresh culture medium was added to inactivate the trypsin, and cells were collected in flow cytometer tubes. The cells were labeled with Anti-ER alpha antibody [SP1] ab16660 at 1/20 dilution, Alexa Fluor® 488 Conjugate, Abcam) according to the manufacturer's instructions (available at: https://www.abcam.com/estrogen-receptor-alpha-antibody-sp1-ab16660.html). Analysis was performed using the BD FACS Calibur flow cytometer and cell sorter and Cell Quest™ software. Unstained control cells were used to determine the percentage of ER-positive cells in the samples.

Statistical analysis:

The data obtained from the applied assays were analyzed for statistical significance using tests for analysis of variance (one-way stacked ANOVA/ 95% confidence) followed by Tukey's Honestly Significant Differences (HSD) multiple range test using GraphPad Prism V.8.0.2 (GraphPad Software, Inc, La Jolla, USA) software.

In all techniques, the mean values for three independent assays and standard deviations were calculated. Differences were recognized statistically significant at P< 0.05.

Results

1- ER-alpha receptor expression:

The immunoreactivity of MCF-7 cells to ER was almost negative throughout the tumor cells. (Fig1)

2- Methylparaben and MTT assay:

There was a non-significant difference between the escalated concentrations of MP compared to untreated MCF7 cells after seven days of incubation. MP exhibited no cytotoxic effect (p>0.1) (Figure 2a, 2b).

3- Cell proliferation assay regulation of proliferation in the absence of ER receptors:

After 7 days, there was a non-significant difference in cell counts with indistinguishable proliferation effects as compared between the MP treated and untreated MCF7 cells. (p>0.1). MP did not stimulate the proliferation of cells even at the highest concentration (200 µM). (Figure 3a, 3b)

4- Quantitative flow cytometry assay for ER- alpha expression:

The α-ER receptor status of MCF7 cells treated with the lowest and highest doses of MP after seven days of incubation showed that MP caused a non-statistically significant expression of ER relative to untreated cells, where the control cells expressed 8.74% only and the concentrations of MP 40 µM and 200µM express 8.21% and 12.52%, respectively. (p>0.1) (Figures 3c,3d)

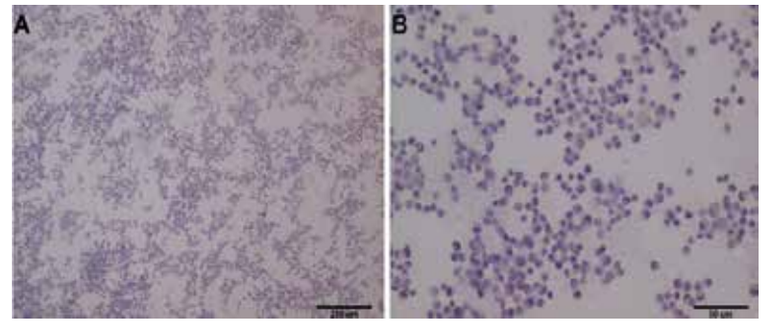

Figure 1. A photomicrograph of negative immunoreaction of MCF-7 cells to estrogen receptor. (ER alpha monoclonal antibody, DAB chromogen with hematoxylin coubterstain. Microscopic magnification; A (×100), Photo B (×400).

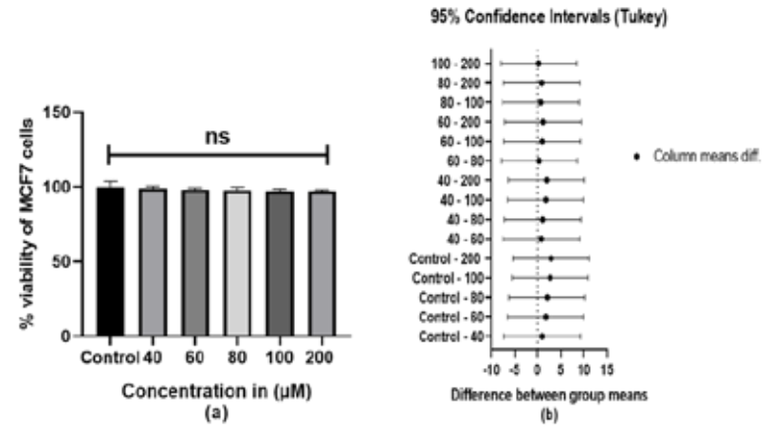

Figure 2. MTT assay after 7 days of incubation of MCF7 cells with different concentrations of methylparaben (MP). (a) Each bar represents the mean ± SD of percentage viability of MCF-7 cells exposed to 40, 60, 80, 100 and 200 µM of methylparaben. ns; non-significant. (p>0.1).
(b) Tukey multiple comparisons test (mean differences) between the various concentrations of MP, If the confidence interval doesn't contain zero this denotes a significant difference.

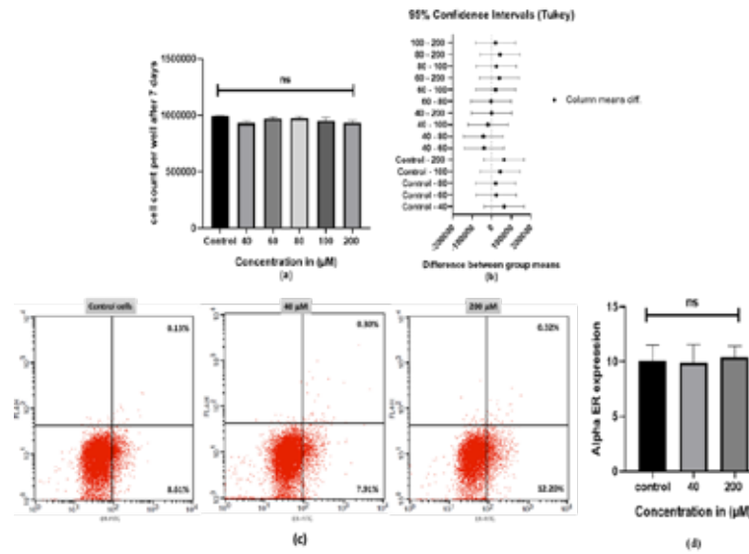

Figure 3. Effects of incremental concentrations of Methyl parabens on proliferation of ER-ve MCF7 cancer cells after 7 days with expression of α-ER receptor by MCF7 cells treated with lowest and highest doses of methyl paraben for 7 days. (a) Each bar represents the mean ± SD of cell count per well of MCF-7 cells exposed to 40, 60, 80, 100 and 200 µM of methylparaben. ns; non-significant. (p>0.1). (b) Tukey multiple comparisons test (mean differences) between the various concentrations of MP, If the confidence interval doesn't contain zero this denotes a significant difference. (c) A representative dot plot of the expression of ER in MCF-7 cells showing the percentage of expression of the gated untreated cells and cells treated with the lowest 40µM and highest concentrations 200µM of methyl paraben. (d) Each bar represents the mean ± SD from three independent experiments; each experiment consisted of triplicates per treatment group. ns; non-significant. (p>0.1).

Discussion

Since the 90s, the human breast adenocarcinoma cell line MCF-7 has served as a standard model for in vitro cancer research as well as endocrine disruptive substances on estrogen-sensitive human cells. MCF-7 cell line has been recognized for its utility for the study of ER-alpha, as this cell line expresses substantial levels of ER, mimicking the majority of invasive human breast cancers that express ER. Since the majority of breast cancers are ER+ [12,13], most studies focused on ER +ve MCF-7 cells.

The estrogenic effects of parabens such as methylparaben, ethylparaben, n-propylparaben and n-butylparaben have been reported in estrogen-sensitive MCF7 human breast cancer cells as well as in other lines of human breast cancer cells [1,14,15]. Additionally, their accumulation in human breast tissue [16,17] indicates their potential to drive the sustained proliferation of ER +ve breast epithelial cells. Parabens are full agonists with the same efficacy as estradiol when available at high concentrations, as it increases proliferation of human breast cancer cells to about the same level, where the maximal cell yield attained was with 3×10^{-11} M. However, at a concentration on the order of 10^5 to 10^7 times higher than that for E2. The main focus, therefore, is not on their low binding affinity to ER, but on the concentration of paraben residing in the target breast tissue [18].

The affinity of methylparaben (MP) for competitive binding to the ERα of MCF7 cells is considered the lowest among the other parabens, and subsequently the magnitude of its inhibitory action on estradiol binding. Yet, the mechanism of MP toxicity is thought to be ER-mediated and linked to the action of estrogen on the cells. The fact was evidenced by studies observing that the proliferative response was inhibited by the addition of antiestrogen [1,14,15]. Similarly, the MP proliferative effect was recorded in the presence of low levels of estrogen, as well as in the absence of estrogen imitating the in vivo conditions after menopause [19].

In 2013, Khanna and Dabre [20] demonstrated the stimulatory effect of MP on non-transformed MCF10A with loss of anchorage dependence, thus evoked the human breast epithelial cells for proliferation in culture suspension. Wróbel and Gregoraszczuk, 2013 [21] have indicated the presence of different mechanisms of the proliferative action of parabens in various investigated cell lines. In estrogen- responsive MCF-7, parabens stimulate their proliferation by increasing estradiol secretion and aromatase activity. Pugazhendhi and Darbre (2010) demonstrated that there were no detectable levels of ERα or ERβ protein in the strain of MCF10A cells and that only over-expression of ERα induced cell growth suspension by estradiol in these epithelial cells. Accordingly, the issue remains a point of research concern to determine whether parabens might act by increasing levels of ERα or whether the mechanism is non-ER-mediated [22].

To determine whether MP could induce human breast cancer growth in ER-ve types, an estrogen-receptor negative MCF-7 cell line was used in this study. Based on literature reviews, the available data from different studies do not provide sufficient information to be able to reach a conclusion.

In the current study, the proliferation of ER-ve MCF-7 cells was unaffected by the presence of MP up to concentrations of 2×10^{-4} M. The loss of ER-alpha resulted in reduced insulin-like growth factor (IGF)-mediated signaling and growth and reduced insulin-like growth factor-I receptor (IGF-IR) and insulin receptor substrate-1 (IRS-1) mRNA and protein expression in comparison with ER +ve MCF-7 cells, and these effects were not inducible by estrogen [23]. In a study by Oesterreich and associates (2001), they demonstrated that the re-expression of ER-alpha in the same cell line restores ER functions (such as signaling and proliferation) as well as the IGF-responsive phenotype, with re-expression of IGF-signaling molecules and growth in response to IGF. Thus, in MCF-7 cells, ER-alpha is a critical regulator of IGF-mediated signaling and growth [23].

Despite the fact that MCF7, employed in previous studies, predominately expresses ER alpha [18], the experimented strain failed to express ER, which could be attributed to cell transformation with loss of featuring receptors [24-26]. It is noteworthy that maintaining expression of ERα in cultured immortalized cell lines is difficult, which resulted in the generation of far more ER-negative than ER-positive human breast cancer cell lines [24]. Such facts explained the failure of the immunocytochemical assay in the current study to detect ER-alpha on the different experimented MCF7 cells.

In agreement with our results, Dabre et al. (2002) utilized triple-negative (MDA-MB-231) human breast cancer cells, which are estrogen receptor-negative cell line, to evaluate the proliferation response of these cells to isobutylparaben. They proved that the MDA MB 231 cells did not proliferate in response to 17β-oestradiol, and therefore isobutyl-paraben failed to influence their growth at concentrations reaching up to 10^{-4} M [14]. These findings provided evidence that the proliferation of cancer breast cells in response to MP is mediated by ERs. Accordingly, the loss of ER-alpha would interfere with the proliferation potentials of MP on breast epithelium [23].

Furthermore, the lack of ER expression indicates both phenotypic and cytogenetic variability for particular clones of breast carcinoma cells [23,26,27]. MCF-7 sub-lines demonstrate a wide divergence in the relative expression of ER, PR and HER2. The proportion of the dominant phenotype may be maintained by growth conditions. For example, the predominance of the ER-positive phenotype could be maintained by the presence of small amounts of estrogen in the fetal bovine serum [27]. To exclude such a conflicting potential, we used charcoal- free FBS in the current study. The latter is free from lipid components, including steroid hormones as estrogen [23,27]. Interestingly, the ER-, PR- and HER2-negative (triple-negative) sub-lines can be originated from the ER-positive MCF-7 cell line [24]. They can be produced by long-term estrogen withdrawal or incubation with fulvestrant, an anti- estrogen, for a prolonged period of time (>12 months) [23,24]. These cell sub-lines might form a useful model for understanding the behavior of triple-negative breast cancers in clinical practice and their response to therapeutic agents in the presence of parabens in general and MP in particular.

Conclusions

The present in vitro study aimed at assessing methylparaben effects on the behavior of ER-ve MCF-7 breast cancer cells. The lack of proliferation of MCF-7 cells was independent of the concentrations used for methylparaben, which confirmed

the ER-dependent effects of methylparaben on these cells. Therefore, confirmation of the sensitivity of MCF7 cell line to estrogen and their characterization in terms of the passage number, genetic diversity of the cells and cross-contamination is recommended before using MCF7 cell line for assessing changes in cancer response to various agents. Moreover, the study of variants of a single cancer cell line might enable recapitulating the behavior of multiple phenotypes of breast cancer in the presence of parabens. These data show that ER is a critical requirement for IGF signaling, and for the functional mechanisms of ERα expression that confers estrogen-mediated growth of an ER-negative breast cancer cell line.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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